Antioxidant and Cytotoxic Activity of *Lentinus fasciatus*

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ABSTRACT

The genus *Lentinus* is one of the most studied and medicinally significant groups among mushrooms. *Lentinus fasciatus* is a considerably understudied species and a methanolic formulation was evaluated for its medicinal potential. Phytochemical analysis unveiled the presence of a high amount of phenolic substances in the methanolic extract of the basidiocarps of *L. fasciatus*. The extracted fraction showed notable scavenging properties in in-vitro antioxidant property estimation assays. In the ABTS and DPPH assay, respectively, the EC_{50} values were 332.12 and 180.78 µg/mL. The mushroom extract was also screened for cytotoxic activity against the human breast adenocarcinoma cells (MCF-7). The biocidal activity against cancer cells is further proven by the low LD_{50} value of 246 g/mL in the WST-1 experiment. The background mechanism behind the cytotoxicity was predicted to be mediated by the apoptotic pathways.

Keywords: Antioxidant property, cytotoxicity, free radicals, methanolic extract, mushroom.

INTRODUCTION

Free radicals are recognized to be potentially harmful chemical entities having one or more unpaired electrons. These electrically charged molecules attempt to neutralize themselves by oxidizing other compounds [1]. ROS is the most abundantly produced by products of metabolic processes [2]. The main target of these reactive molecules includes nucleic acids, proteins, lipids, and structural carbohydrates. ROS causes oxidation of proteins and RNA, lesions on DNA strands, alterations in polyunsaturated fatty acids in membranes, mitochondrial depolarization, apoptosis, and many more deleterious effects [3]. A proper balance between the production of oxidants and antioxidants is maintained in healthy living beings. An unhealthy lifestyle, environmental pollution and other factors are forcefully disrupting this balance, for which dietary supplementation of antioxidants is becoming a necessity these days.

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butyl hydroquinone (TBHQ), propyl gallate (PG) are some of the widely used chemically synthesized antioxidants in the food and medicine industry [4]. Although synthetic antioxidants are in extensive use, safety issues have arisen over time. Long-term intake of these substances can cause major health problems and, in certain circumstances, increase the risk of cancer. BHT has already been shown to be carcinogenic at higher dosages.[5] Thus, there is a growing demand for naturally derived antioxidants with less side effects [6].

Mushrooms have been known to be a treasury of natural bioactive compounds and usage of mushrooms for therapeutic purposes dates back to the Neolithic age. Decades of research have established mushrooms are rich in different kinds of bioactive phytochemicals that are slowly being proved to be effective against several human ailments [6–9]. Most of the species among the genus Lentinus are edible but investigation on their medicinal properties is scarce. Recently, an edible mushroom *Lentinus fasciatus*

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Received: 24/1/2022 Accepted:19/6/2022.
DOI: https://doi.org/10.35516/jjps.v16i1.1064

was collected from West Bengal, India [10], whose medicinal importance has not been widely explored. In this article, we have reported the mycochemical composition, antioxidant and cytotoxic efficacy of the methanolic extract of *Lentinus fasciatus*.

MATERIAL AND METHODS

Sample Preparation

Fresh fruit bodies of *Lentinus fasciatus*, naturally grown on the roadside wooden logs, were collected from Barasat, West Bengal, India. The mushroom was identified and authenticated using standard literature [10]. Collected basidiocarps were dried at 40°C for 48h and grinded using an electronic grinder and passed through 160 mesh and stored in a sealed container.

Extraction

Five grams of powdered sample was extracted with methanol for 24h with agitation at regular intervals and this was repeated twice with the residue. The methanolic extract was evaporated to dryness and redissolved in dimethyl sulfoxide (DMSO) to get a stock solution of 500 mg/mL. The stock solution was dissolved further, according to the requirement of the experiments.

Phytochemical analysis

Folin-Ciocalteau reagent was used to measure the total phenolic content [11]. Total phenolic content is represented as µg of gallic acid equivalent (GAE) / mg of extract. Total flavonoid was evaluated according to a customary protocol [12] followed by comparing it with quercetin standard curve. Similarly, the flavonoid content is expressed as µg of quercetin equivalent (QE) / mg of extract. For estimation of β-Carotene and lycopene content 100µL of the extract was taken in a test tube and mixed with 10 mL of acetone and hexane (4:6). The absorbance of the mixture was measured at 453, 505 and 663 nm and the β-Carotene and lycopene content were estimated using the given formula (Nagata and Yamashita, 1992).

$$\beta - \text{Carotene (mg/100mL)} = (0.0458 \times A_{663}) + (0.373 \times A_{505}) - (0.0806 \times A_{453})$$

$$\text{Lycopene (mg/100mL)} = (0.216 \times A_{663}) - (0.304 \times A_{505}) + (0.452 \times A_{453})$$

Estimation of Antioxidant Potential

The total antioxidant capacity of the methanolic extract was estimated according to the following protocol [14]. Total antioxidant capacity is indicated in form of µg of ascorbic acid equivalent (AAE) / mg of extract. To assess the DPPH free radical quenching activity, the methodology described by Pereira et. al. was followed with slight modifications [15]. Reactions were carried out on 96 well plate and the reaction volume was kept at 200 µL. Reaction mixtures consisting of gradually increased concentration of the sample extract and DPPH solution were incubated for 30 minutes. The absorbance of the mixtures was checked at 517nm using a microplate reader (iMark™ Microplate Absorbance Reader, BIO-RAD, USA) to calculate the scavenging activity. EC_{50} value was determined which specifies the concentration of extract at which 50% scavenging of the free radicals took place. ABTS free radical scavenging activity of the extracted fraction was determined according to the protocol standardized by Khatua et. al. (2013) [16]. Similar to DPPH free radical scavenging assay the EC_{50} value was computed from the graphical curve. For authentication, the obtained data of DPPH and ABTS assay was compared to ascorbic acid and trolox respectively.

Cell Culture

Human breast adenocarcinoma cell line MCF-7, obtained from the cell line repository at National Centre for Cell Science, Pune was maintained in Minimum essential medium (***MEM***) supplemented with 10% Foetal Bovine Serum. For optimal growth, the cells were incubated in a humified incubator (CO_2 Incubator, Esco Micro Pte Ltd, Singapore) at 37 °C with 5% CO_2. Trypsin-EDTA was used to dissociate the confluent cells and the

cells were seeded in variable plates for experiments and incubated for 24 hours. The amount of DMSO was restricted to <1% during treatment.

Invitro cytotoxicity assay

MCF-7 cells were seeded in a 96-well microtiter plate and incubated for 24 hours to obtain the cells at the log phase. Treatment of different concentrations of the extract was given to the cells and again incubated for 24h. After that, 10 µL WST-1 reagent (TaKaRa, Japan) was added to each well containing the treated cells and incubated for 2h. Absorbance was measured according to the manufacturer's protocol using a plate reader. The reduction in absorbance was analyzed to determine the cell death percentage at different concentrations and LD_{50} value.

Detection of Apoptosis

Treated cells were stained with acridine orange (AO, 5 mg/mL) and ethidium bromide (EB, 3 mg/mL) and photographed under a fluorescence microscope (Floid Imaging Station, Life Technologies, Waltham, MA, USA). Dead cells produced a brilliant orange fluorescence due to the uptake of EB, while the live cells appeared green due to the uptake of AO.

To further confirm the nature of cell death, nuclear morphology was also characterized using the DAPI staining methodology. After washing the treated cells with phosphate-buffered saline (PBS), the cells were treated with 6-diamidino-2-phenylindole (DAPI) (1µg/mL in PBS). After 15 minutes the washed cells were observed under a fluorescence microscope for nuclear abnormalities.

Statistical analysis

All data are exhibited as the mean ± SD of "n" independent measurements as indicated in the corresponding figure legends. Statistical comparisons were calculated using Student's t-test. A value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Crude organic extracts contain a complex mixture of phenolic compounds which are soluble only in certain organic solvents. A literature survey has confirmed that methanol extraction is one of the most efficient extraction procedures to pull out the phenol-rich fraction from mushroom samples as Methanol represents a higher polarity index than ethanol, ethyl acetate, acetone and other organic solvents [17].

Phytochemical analysis

In this study, methanolic extract of *Lentinus fasciatus* was prepared to quantify some of the significant bio-active chemical groups to confirm the medicinal potential of this mushroom. A yellowish-brown organic formulation was obtained with a high extractive yield of 12.51%. The prime chemical group in the methanolic extract of *L. fasciatus* was found to be phenol. It is a well-established phenomenon that phenolic compounds tend to reduce potential oxidant species due to the presence of the aromatic ring. Besides their antioxidant properties phenols are good antibacterial, antiviral, anti-carcinogenic and anti-mutagenic agents [18]. β-carotene and lycopene are the two main fungal carotenoids, known for their antioxidant activities [19]. Data validates that phenols and flavonoids are the key components that are putative antioxidants, whereas β-carotene and lycopene were found in trace amounts. The total phenol, flavonoid, β-carotene, and lycopene content is summarised in Table 1.

Table 1: Proximate phytochemical composition of the methanol soluble fraction of *L. fasciatus.*

Extractive yield	Total phenols (µg GAE / mg of extract)	Flavonoids (µg QE / mg of extract)	β-Carotene (µg/mg of extract)	Lycopene (µg/mg of extract)
12.51 %	60.894 ± 2.29	5.54 ± 0.84	0.1344 ± 0.006	0.0987 ± 0.006

Antioxidant assays

Antioxidants can counteract various types of radicals or oxidants in several different ways. Thus, to evaluate the antioxidant potential of the methanolic extract of *L. fasciatus*, a series of assays were performed. Phosphomolybdenum method gives a good measure of the antioxidant activity of crude extracts. Mo(IV) is reduced to Mo(V) by antioxidant species in acidic pH and a green phosphate/Mo(V) compound with absorption maxima at 695nm is formed [14]. By spectrometric analysis, the total antioxidant capacity of the methanolic extract was found to be 36.025 ± 0.601 µg AAE/ mg of extract.

ABTS assay is one of the simplest and reliable assays to assess the antioxidant property of biological samples. $ABTS^{\bullet+}$ is produced by oxidizing ABTS with potassium persulfate. The dark bluish-green colour is neutralized upon reduction of $ABTS^{\bullet+}$ by hydrogen donating antioxidants and the measurement of photometric change gives an accurate indication of the antioxidant activity of the sample [20]. The experimental extract was found to be a notable scavenger as it showed quite a significant scavenging activity (18.42%) even at a dose as low as 100 µg/mL. Further, the percentage of scavenging was extended to 34.71, 45.64, 59.70, 70.73 in the presence of the extract at 200, 300, 400, 500 µg/mL respectively (Figure 1). Effective concentration (EC_{50}) at which 50 % scavenging took place was found to be 332.122 ± 0.838 µg/mL.

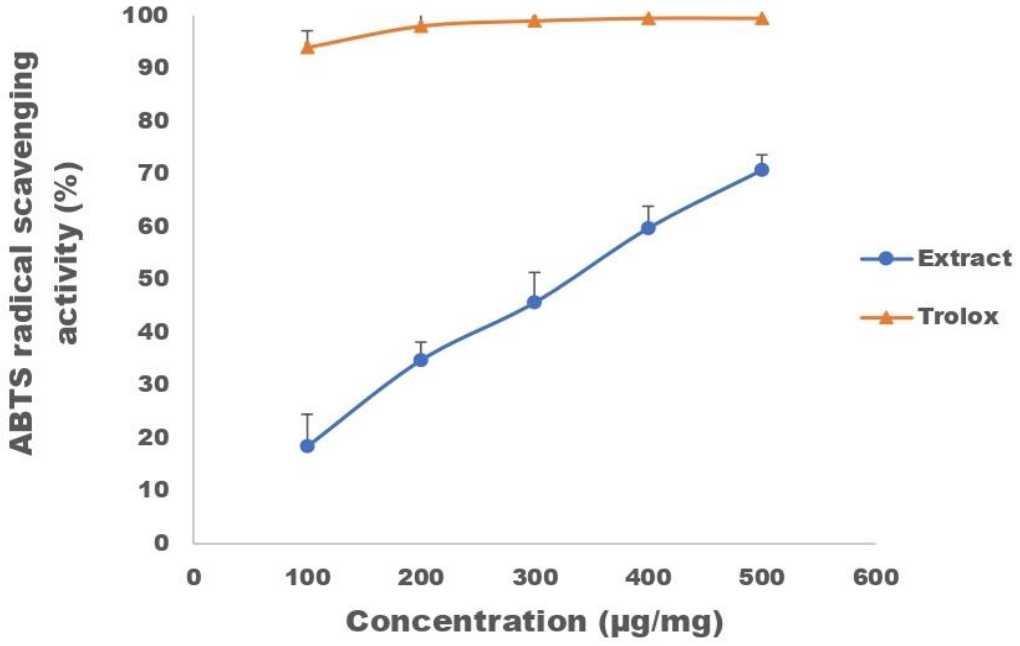

Figure 1: ABTS free radical scavenging activity of the methanolic extract of *L. fasciatus* compared to ascorbic acid as standard. Data are presented as mean ±SEM and are representative of three independent experiments. $p < 0.05$.

Moreover, in order to visualize the scavenging activity in a better way, the extract was tested against DPPH, a commercially available stable free radical that is commonly used to assess the antioxidant property of test samples. The purple colour of the free radical solution gradually fades upon reduction of DPPH radicals by the reducing agents such as antioxidants [15]. The loss of colour gives a photometric measurement of the free radical scavenging property of the test samples. Methanolic extract of *L. fasciatus* showed considerable scavenging property that surged up to 77.1 % at the higher dose of 300 µg/mL (Figure 2). EC_{50} value against DPPH was found to be 180.78 ± 4.04 µg/mL which is lower than the published reports [21] of other *Lentinus* species like *L. squarrosulus*, *L sajor-caju*. The extracted fraction of the mushroom showed potential antioxidant activity *in-vitro*, indicating that it is an effective antioxidant (Table 2).

Table 2: Antioxidant and Cytotoxic activity of methanolic extract of *L. fasciatus*

Total Antioxidant Capacity	EC_{50} value for ABTS free radical scavenging	EC_{50} value for DPPH free radical scavenging	LD_{50} value of Cytotoxic activity against MCF-7 cells.
36.025 ± 0.601 µg Ascorbic Acid Equivalent (AAE)/ mg of extract	332.122 ± 0.838 µg/mL	180.78 ± 4.04 µg/mL	246 ± 1.045 µg/mL

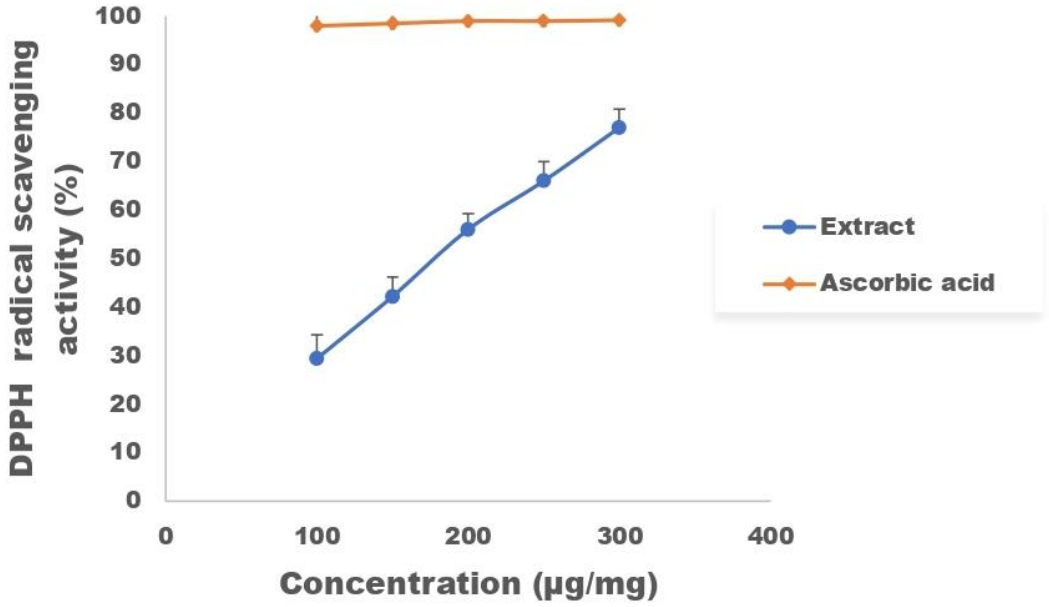

Figure 2: DPPH radical scavenging activity of the methanolic extract of *L. fasciatus* compared with standard. Data are presented as mean ±SEM and are representative of three independent experiments. $p < 0.05$.

Cytotoxicity assessment

Extensive research established that there is a close correlation between oxidative stress and cancer. Excessive generation of reactive oxygen species stimulates the uncontrolled growth of cells leading to the development of tumours and initiating the process of carcinogenesis [22].

Furthermore, oxidative stress weakens the body's innate antioxidant defence system against angiogenesis and metastasis [23]. Therefore, after getting a positive response in the antioxidant assays, the extracted fraction was further screened for cytotoxic effects on a breast cancer cell line.

To assess the cytotoxic effects of the extract the treated MCF-7 cells were stained with WST-1 regent and orange-colored formazan was produced in proportion to the presence of living cells. WST-1 assay aided us to calculate the proportion of cells that died at various doses of the extract and determining its LD50. At 500 g/mL, 82.56 percent of cancerous cells died, evidencing that the extract is effective in killing cancerous cells (Figure 3). The LD50 value was obtained to be 246 ± 1.045 µg/mL.

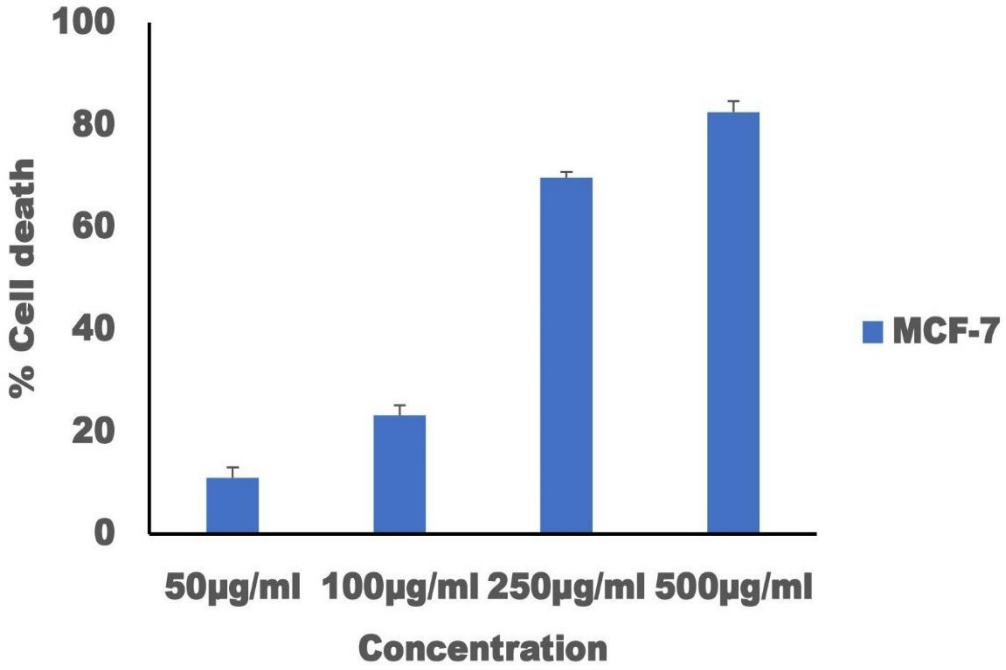

Figure 3: Percentage of cell death in MCF-7 cells induced by the methanolic extract of *L. fasciatus*. Data are presented as mean ±SEM and are representative of three independent experiments. p < 0.05.

To visually differentiate between live and dead cells, dual staining with EB and AO was executed. AO can penetrate all the cells independent of their viability but EB cannot make their way into the cells until the integrity of the plasma membrane and the nuclear membrane is lost downstream of death signals [24]. The majority of the treated cells showed orange fluorescence due to the intercalation of EB into their DNA, whereas all cells in the control set were fluorescing only green (Figure 4A). Loss of membrane integrity is also a hallmark of induction of apoptosis.

Apoptosis or programmed cell death is a pathway that entails a series of phenotypic changes in the cell undergoing apoptosis, among which one of the most important attributes being nuclear shrinkage and disintegration [25]. To confirm whether the cytotoxicity imparted by the extract is by inducing apoptosis, DAPI, a fluorescent dye that intercalates into the grooves of DNA, was used to stain the nucleus. Nuclear deformities including shrinkage, blebbing and fragmentation were

observed in most of the treated cells whereas untreated cells portrayed uniform chromatin staining. The observed changes in the nucleus (Figure 4C) indicates that the cell death was possibly mediated by the apoptotic pathway.

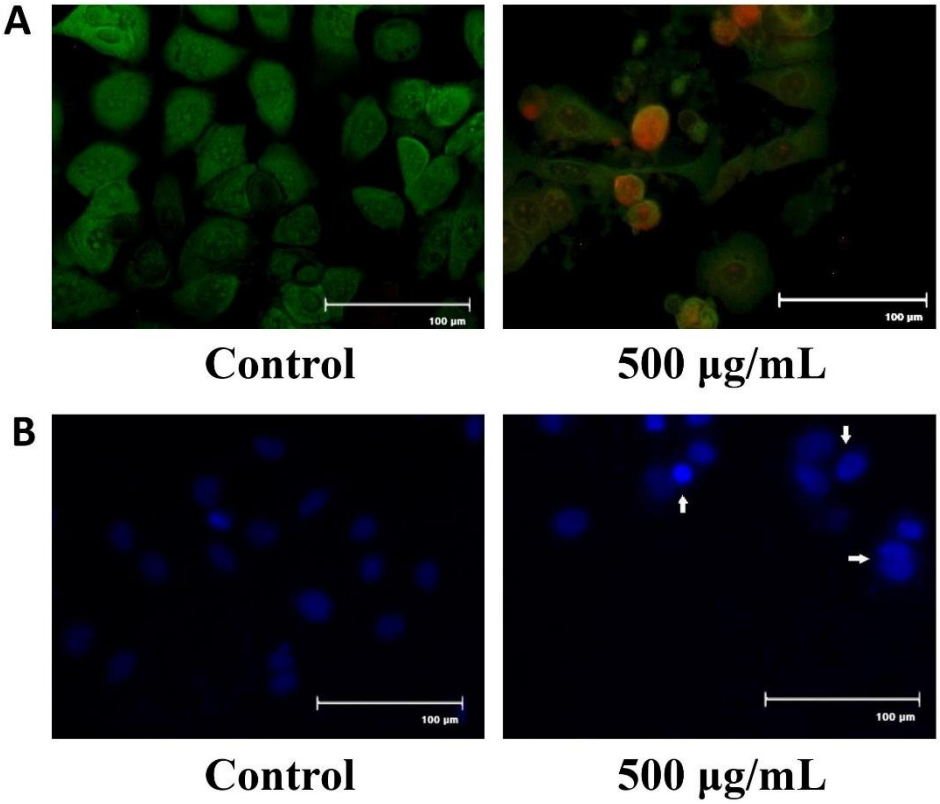

Figure 4: (A) Representation AO/EB dual stained cells after treatment for 24h. (B) DAPI stained cells after treatment for 24h. Images taken under 20X magnification, representing the best of the replicates (n = 3).

CONCLUSION

The methanolic extract of *Lentinus fasciatus* has considerable antioxidant properties, as shown in the findings of this investigation. The crude extract is rich in antioxidant components including phenol and flavonoids as revealed by the quantitative phytochemical assessments. In addition, the cytotoxic property against human cancer cell line displays potential as a possible anti-cancer formulation. Although an early indication of apoptosis was observed in the cancer cells, the underlying mechanism needs further validation. Overall, this mushroom can become an important addition to the future pharmaceutics.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the Department of Biotechnology (DBT), Government of India and

Department of Botany, University of Calcutta, for providing necessary funds and instrumental assistance.

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نشاط مضادات الأكسدة والسمية للخلايا في *Lentinus fasciatus*

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ملخص

يعتبر جنس Lentinus من أكثر المجموعات التي تمت دراستها وذات الأهمية الطبية بين عيش الغراب. Lentinus fasciatus هو نوع غير مدروس بشكل كبير وتم تقييم تركيبة ميثانولية لإمكاناته الطبية. كشف التحليل الكيميائي النباتي النقاب عن وجود كمية عالية من المواد الفينولية في المستخلص الميثانولي من الباسيدوكاربس من L. fasciatus. أظهر الجزء المستخرج خصائص كسح ملحوظة في مقايسات تقدير خصائص مضادات الأكسدة في المختبر. في اختبار ABTS و DPPH، على التوالي، كانت قيم EC50 332.12 و 180.78 ميكروغرام / مل. تم فحص مستخلص الفطر أيضًا بحثًا عن النشاط السام للخلايا ضد خلايا سرطان الثدي البشري (MCF-7). تم إثبات نشاط المبيدات الحيوية ضد الخلايا السرطانية من خلال قيمة LD50 المنخفضة البالغة 246 جم / مل في تجربة WST-1. تم توقع آلية الخلفية وراء السمية الخلوية بواسطة مسارات موت الخلايا المبرمج.

الكلمات الدالة: خاصية مضادات الأكسدة، السمية الخلوية، الجذور الحرة، خلاصة الميثانول، الفطر.

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تاريخ استلام البحث 2022/1/24 وتاريخ قبوله للنشر 2022/6/19.